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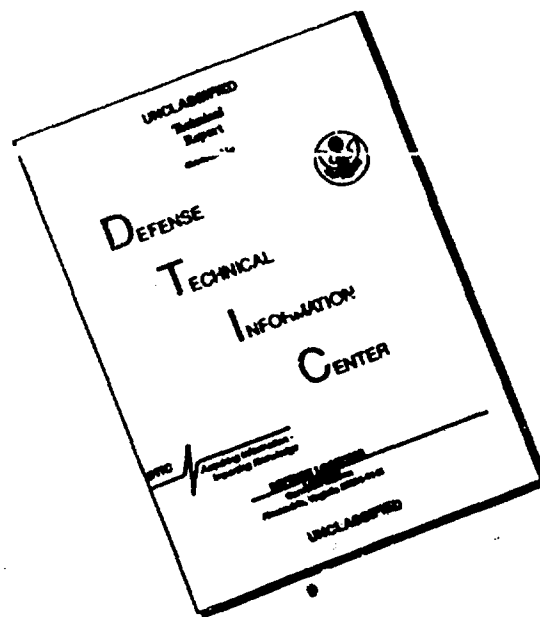
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Random amplified polymorphic DNA (RAPD) markers readily distinguish cryptic mosquito species (Diptera: Culicidae: *Anopheles*)

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Abstract

The usefulness of random amplified polymorphic DNA (RAPD) was examined as a potential tool to differentiate cryptic mosquito species. It proved to be a quick, effective means of finding genetic markers to separate two laboratory populations of morphologically indistinguishable African malaria vectors, *Anopheles gambiae* and *An. arabiensis*. In an initial screening of fifty-seven RAPD primers, 377 bands were produced, 295 of which differed between the two species. Based on criteria of interpretability, simplicity and reproducibility, thirteen primers were chosen for further screening using DNA from thirty individuals of each species. Seven primers produced diagnostic bands, five of which are described here. Some problematic characteristics of RAPD banding patterns are discussed and approaches to overcome these are suggested.

Keywords: RAPD, *Anopheles*, random primers.

Introduction

Mosquitoes transmit more human parasitic diseases than any other arthropod group. By one estimate, *Anopheles* mosquitoes alone are responsible for nearly 500 million clinical cases of malaria each year (Sturchler, 1989). Many of the most important malaria vectors are members of morphologically indistinguishable or similar species complexes, e.g. the *maculipennis* group (Guy *et al.*, 1976; White, 1978); the *quadrimaculatus* complex (Mitchell *et al.*, 1993); the *dirius* complex (Peyton & Ramalingam,

1988); and the *gambiae* complex (White, 1985). These cryptic species have often confused epidemiological, ecological and taxonomic research. To facilitate identification of cryptic mosquito species, researchers have employed a wide range of cytological and biochemical approaches, in addition to traditional morphological comparisons (Service, 1988). These include analysis of chromosome structure and genetic compatibility (Fritz *et al.*, 1991), comparison of allele frequencies by protein electrophoresis (Narang *et al.*, 1989), immunology (Ma *et al.*, 1990), DNA hybridization (Cockburn, 1990), mitochondrial DNA (mtDNA) and ribosomal DNA (rDNA) restriction fragment length polymorphism (RFLP) analysis (Collins *et al.*, 1988; Mitchell *et al.*, 1993), and rDNA sequence comparison (McLain & Collins, 1989). Some of these techniques lack sufficient resolving power to answer questions at the species or population level, and all are labour and time intensive, requiring laboratory facilities and specialized technical expertise not available to most insect systematists. The ability to amplify DNA via the polymerase chain reaction (PCR) has greatly facilitated DNA sequence comparisons (e.g. Innis *et al.*, 1990) and resulted in the development and use of species diagnostic PCR primer pairs (Paskewitz & Collins, 1990; J. Scott, W. Brogden and F. Collins, pers. comm.). Even though this is an important application of PCR technology, it still requires extensive preliminary sequence information for characterization of the taxa under consideration.

Recently, Williams *et al.* (1990) and Welsh & McClelland (1990) described a novel means of obtaining genetic markers which is not dependent on *a priori* sequence information, and which may be technically accessible to a wider range of entomologists. This technique, random amplified polymorphic DNA (RAPD) is PCR based, permitting scores of markers to be assayed on DNA extracted from a single mosquito. Instead of using primer pairs as in traditional PCR, RAPD reactions use a single short primer (usually ten bases in length) of randomly chosen sequence. For a RAPD band to be produced, the primer needs to match a binding site that is within approximately 2–3 kilobase pairs of another, oppositely oriented binding site, so that the single oligonucleotide can prime replication in both the forward and reverse direction. A typical RAPD reaction produces multiple amplification products, each representing a discrete genetic locus, which can be analysed easily by agarose gel electrophoresis. RAPD bands

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may display a high degree of polymorphism, and screening multiple primers against taxa of interest has proven to be a means of quickly identifying species-specific markers (Arnold *et al.*, 1991). Additionally, RAPD markers derive from multiple loci and have the potential to provide important information on mosquito population genetic structure that would not be available from a single locus marker. Since its development, RAPD has shown promise for use in a wide variety of organisms including bacteria, higher plants, vertebrates and invertebrates, including mosquitoes and other insects, as a tool for genetic mapping, strain identification and systematics (Williams *et al.*, 1992; Bowditch *et al.*, 1992; Hadrys *et al.*, 1992; Chapco *et al.*, 1992; Black *et al.*, 1992; Kambhampati *et al.*, 1992; Perring *et al.*, 1993).

To evaluate the potential of RAPD reactions to produce diagnostic markers for analysing cryptic species complexes of mosquitoes, and presumably other dipterans, we applied it to two morphologically indistinguishable taxa within the *Anopheles gambiae* complex, *An. gambiae* Giles and *An. arabiensis* Patton. Historically, these taxa have been distinguished by polytene chromosome banding, a time-consuming and difficult technique available only to specialists. We report here the results of screening fifty-seven RAPD primers on colony-maintained population samples of the two species. We discuss the types of RAPD patterns observed in our survey and suggest ways to avoid some of the problems which may be encountered in RAPD analyses. RAPD analysis proved highly effective in separating the two species, and molecular markers to identify these epidemiologically important malaria vectors were quickly obtained. Since these data derive from colony maintained specimens, they may or may not apply to wild populations; however, we felt it necessary to control the source and identity of specimens for this initial study.

Results

As a primary screen for RAPD markers, PCR was carried out under standard conditions (see Experimental Procedures below) for fifty-seven random ten-base primers on DNA samples from *An. gambiae* and *An. arabiensis*. Each DNA sample was a pooled population sample from five individual larvae of the respective species. Primers which appeared to produce diagnostic bands were then submitted to a second round of screening. These primers were applied to DNA samples from thirty individual mosquito larvae from each species as a test of their diagnostic ability, and to investigate the amount of genetic variability within the sampled populations.

Each of the fifty-seven primers produced multiple amplification products. Because RAPD reactions often produce a pattern of bright bands together with fainter bands or faintly smeared regions in the gel, complex patterns of faint

bands can be difficult to compare between two species. Furthermore, since faint bands, especially those of higher molecular weight, exhibit inconsistent amplification from the same sample, we chose to score only bands which were bright, distinct, and, in our experience, likely to be reproducible. The fifty-seven primers produced 210 scorable bands for the pooled *An. gambiae* sample (3.7 bands/primer) and 249 bands (4.4/primer) for the pooled *An. arabiensis* sample. A total of 377 bands were scored for the two species, of which eighty-two were present in both species. 128 bands were unique to *An. gambiae* and 167 were unique to *An. arabiensis*. Only five primers gave apparently identical patterns of scorable bands for the two species. Twenty-one of the primers (37%) produced PCR profiles with no bands in common between *An. gambiae* and *An. arabiensis*, and almost all other primers gave bands which could potentially serve as markers for these species. Primers which produced complex or poorly resolved banding patterns were not characterized further, even if the patterns were quite different between the two species.

A problem which must be faced in interpreting RAPD banding patterns is that of assessing homologies of bands which appear to be of similar size in both species. This is especially true when a large number of bands is produced, or when the bands in question are of different intensity. For this reason, primers chosen for further screening were those which produced a small number of intense, diagnostic bands. Figure 1A shows typical results from RAPD reactions on the pooled DNA samples and illustrates the rationale for including particular primers in the second round of screening. Primer P1 was rejected because numerous weak bands were produced in both species and some of the bands are shared; P2 was accepted because of several strong bands, all of which differ between species; P4 was accepted because of two strong bands in one species and one in the other which differ between species; P5 was rejected because, while there are strong bands in each species, there are weak ones of the same apparent size in the other; P6 was rejected because both species have many weak bands.

In all, twenty-one primers were considered to meet the criteria described above, and a subset of thirteen of these were applied to DNA samples from thirty individuals of each species. Seven of these primers functioned in a completely diagnostic manner for the colony populations, giving species-specific banding patterns for each individual tested. The other primers failed as markers because the bands which appeared diagnostic on the pooled DNA samples proved to be present in both species when applied to larger population samples. The banding patterns of five of the RAPD markers are discussed below as examples of the results which may be expected in RAPD analysis.

Primer B10 (Table 1; Fig. 1B). This primer produced a

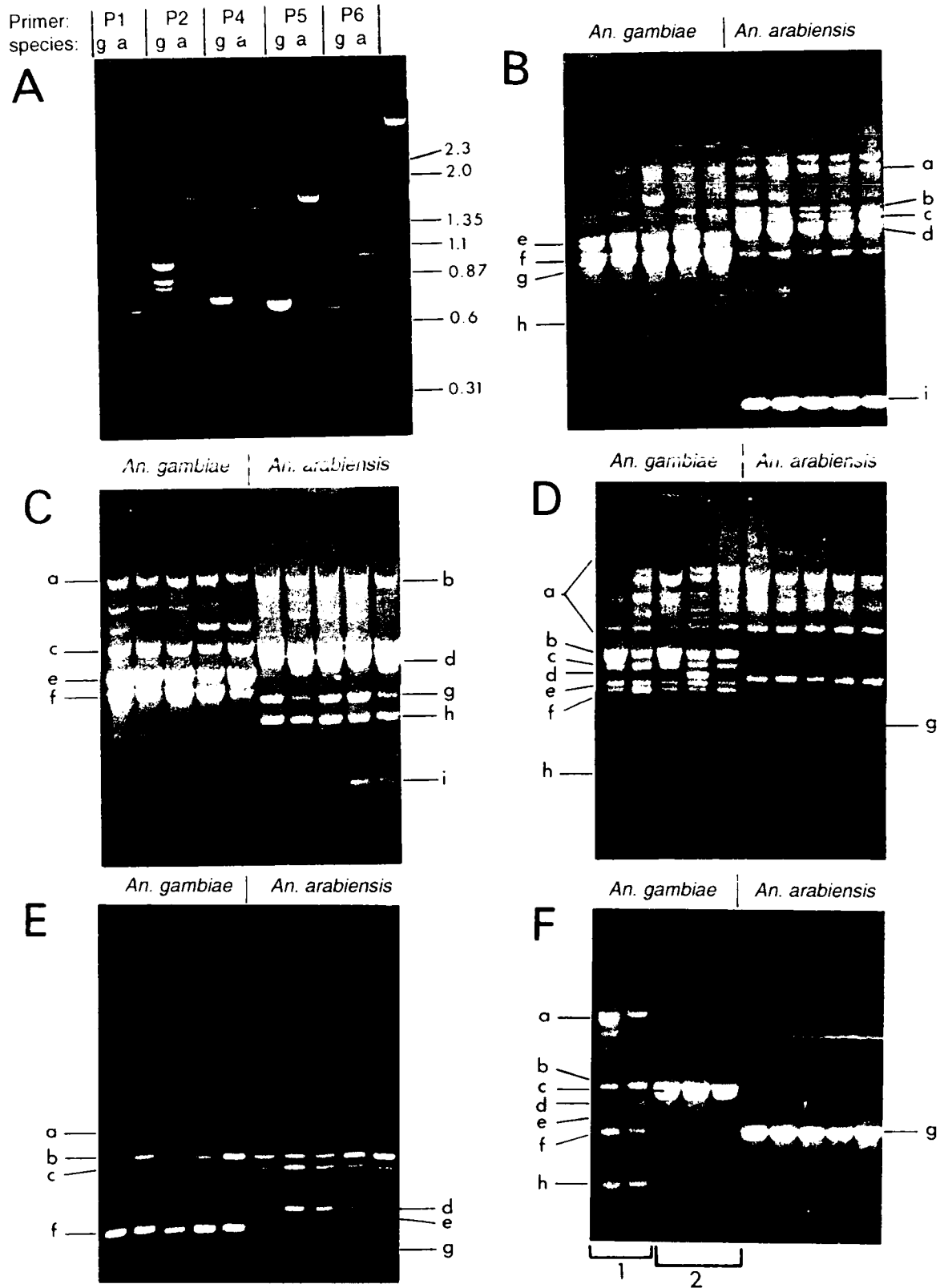


Figure 1. (See text for explanation) (A) Example of primer screening g = *An. gambiae*, a = *An. arabiensis*. DNA size standards on right in kbp. (B) Primer B10. (C) Primer A7. (D) Primer B7. (E) Primer P14. (F) Primer P37, patterns 1 and 2 indicated, see text for explanation.

number of diagnostic bands as well as a species-specific pattern of band intensity. Band 'i' is seen only with *An. arabiensis* samples and bands 'e' and 'g' are seen only with *An. gambiae* (bands 'f' and 'g' appear as one band in this gel but were distinct in others run for longer periods). Bands 'b', 'c' and 'd' form a distinct group of fairly bright bands present in *An. arabiensis* and 'f' is a bright band found in *An. gambiae*. However, these four bands are all represented in the corresponding species by unscorable, weak and/or inconsistent bands. Bands 'a' and 'h' are examples of bands found in both species which are weak, but in this case scorable because they are reproducible.

Primer A7 (Table 1; Fig. 1C). All lettered bands appear to be species-specific except 'c' which is shared by both species. Band 'd' was reproducibly absent in 10% (three of thirty) of *An. arabiensis*, revealing an intraspecific polymorphism that is not present in *An. gambiae*. In contrast, bands 'e' and 'f' were initially found to be missing in two individuals of *An. gambiae*, but when reactions were run again on

these individuals, the bands were present. This underscores the necessity to verify the reproducibility of RAPD banding patterns, especially in instances where inferences might be drawn from negative data, i.e. the absence of bands.

Primer B7 (Table 1; Fig. 1D). This primer gave several completely diagnostic bands ('b' and 'e' for *An. gambiae*; 'g' for *An. arabiensis*) as well as two bands ('c' and 'f') that are usually present in *An. gambiae* (90%), but completely absent in *An. arabiensis*, and 'd' which is always present in *An. arabiensis* and sometimes present in *An. gambiae*. The bands grouped under 'a' are an example of a complex pattern for which homologous bands can not reliably be assigned in the two species, and which were therefore not analysed. Band 'h' is uniformly present in both species.

Primer P14 (Table 1; Fig. 1E). Band 'd' is diagnostic for *An. arabiensis* and 'f' for *An. gambiae*. In addition, bands 'e' and 'g' are found only in *An. arabiensis* but only in 67% of the individuals. Bands 'a', 'b' and 'c' are reproducible bands found in both species.

Primer P37 (Table 2; Fig. 1F). This primer produces one or the other of two distinct patterns for *An. gambiae* and a strong diagnostic band, 'g', in *An. arabiensis*. Pattern 2 is the most common of the two *An. gambiae* patterns, producing a single strong band, 'c', in 83% of the individuals ($n = 40$). Pattern 1 has six weak but consistent bands ('a', 'b', 'd', 'e', 'f' and 'h'). The possibility that the two patterns seen in *An. gambiae* might be sex specific was examined by testing DNA samples from five adult mosquitoes of each sex. No correlation of the patterns with sex was observed (data not shown).

It is interesting that primer P37 produces two completely dissimilar banding patterns among individuals of *An. gambiae*, when all other primers produced similar, if not identical, patterns among individuals within this species. If the presence of RAPD bands were determined solely by the presence of appropriate primer binding sites, the completely dissimilar patterns seen with P37 would suggest a large sequence divergence between the two *An. gambiae* types, inconsistent with the similarity seen with other primers. To gain a better feel for the genetic differences that underlie the two patterns, a competition experiment was carried out with mixtures of template DNA from the two pattern types. DNA samples that normally produced either pattern 1 or pattern 2 were mixed 1:1, 1:2 and 1:10 (ratio of pattern 2-type to pattern 1-type) and the mixed samples subjected to RAPD PCR using primer P37. The strong band 'c' of pattern 2 dominated all reactions, and only when pattern 2-type DNA was diluted 1:10 could bands from pattern 1 be faintly seen (results not shown). Given these results, it is reasonable to suppose that the target sequences characterizing pattern 1 do, in fact, exist in individuals which give RAPD banding pattern 2 (band 'c'), but that the PCR so strongly favours the amplification of band

Table 1. Summary of presence of scorable RAPD fragments produced by selected random primers used to distinguish *An. gambiae* from *An. arabiensis* (Fig. 1B-E) ($n = 30$).

Primer	Band	Approx. m.w. (kbp)	<i>An. gambiae</i> (%)	<i>An. arabiensis</i> (%)
B10	a	1.8	100	100
	b	1.26	0*	100
	c	1.21	0*	100
	d	1.07	0*	100
	e	1.0	100	0
	f	0.89	100	0*
	g	0.87	100	0
	h	0.5	100	0
	i	0.27	0	100
A7	a	2.38	100	0
	b	2.14	0	100
	c	1.21	100	100
	d	1.06	0	90
	e	0.95	100	0
	f	0.84	100	0
	g	0.80	0	100
	h	0.71	0	100
	i	0.44	0	100
B7	a	1.1-2.0	—	—
	b	0.98	100	0
	c	0.91	90	0
	d	0.81	30	100
	e	0.79	100	0
	f	0.76	90	0
	g	0.59	0	100
	h	0.39	100	100
	i	—	—	—
P14	a	1.18	100	100
	b	0.98	100	100
	c	0.91	53	67
	d	0.67	0	100
	e	0.64	0	67
	f	0.58	100	0
	g	0.50	0	67

*Not considered scorable. Faint amplification product sometimes visible but at greatly reduced level compared to the other species.

Table 2. Summary of scorable RAPD fragments produced by primer P37 used to distinguish *An. gambiae* from *An. arabiensis* (Fig. 1F) ($n = 40$).

Primer	Band	Approx. m.w. (kbp)	<i>An. gambiae</i> (%)		<i>An. arabiensis</i> (%)
			Pattern 1 (7/40)	Pattern 2 (33/40)	
P37	a	1.67	100	0	0
	b	0.88	100	0	0
	c	0.83	0	100	0
	d	0.76	100	0	0
	e	0.68	100	0	0
	f	0.62	100	0	0
	g	0.61	0	0	100
	h	0.40	100	0	0

'c' that pattern 1 bands are not visibly produced. We conclude that one cannot always infer that the absence of particular RAPD bands indicates a lack of appropriate primer binding sequences in the template DNA, and that RAPD markers, while usually independent, can compete in the amplification process so that kinetically favoured products predominate.

Discussion

In this application of RAPD analysis to morphologically cryptic mosquito species, the technique proved to be an efficient means to obtain diagnostic molecular markers for laboratory colonies of the two species, with a large majority of primers producing patterns with potential to serve as markers. The frequency of diagnostic primers observed in our survey is quite high in comparison to studies of closely related species pairs from other taxonomic groups (e.g. birds; D. Albright and M. Braun, unpublished data), where dozens of primers may need to be surveyed before potential species-specific markers are discovered. There are no currently accepted methods for estimating genetic divergence from RAPD data and there may be considerable technical difficulties with doing so. However, the genetic dissimilarity suggested by our RAPD survey of two *Anopheles* species is seemingly at odds with their extreme morphological similarity, which would suggest a very close relationship, and a relatively recent divergence. The possible explanations for this include accelerated DNA sequence evolution and/or extremely conservative morphological evolution. Founder effect during the establishment of the laboratory colonies could theoretically have contributed to the observed genetic dissimilarity. The discovery of several polymorphisms in our RAPD analyses (Table 1) argues against severe founder effect, but it cannot be excluded without a survey of wild populations of the two species.

While RAPD analysis is clearly an approach of merit for studies such as ours, we encountered a number of characteristics of RAPD reactions which require caution in the development and application of markers. First, RAPD band

patterns must be empirically determined to be reproducible before their use as markers is justified. Even fairly strong RAPD bands will occasionally fail to be produced in a particular amplification, so inferences based on the absence of a band should be made only after repeated reactions confirm the absence as reproducible. Second, the template competition experiment performed with primer P37 demonstrates that bands may fail to be produced when PCR conditions favour the production of competing amplification products, even when the appropriate primer binding sites are present. Possible reasons why reaction products from one target sequence may be preferentially amplified relative to others which are present in the reaction are: (1) the size of the fragment; (2) extent of primer mismatch, if any; (3) secondary structural characteristics of the single stranded template; and (4) copy number of the target sequence. In the case of primer P37 for *An. gambiae*, band 'c' of pattern 2 may derive from repetitive DNA sequences with a primer binding site that is lacking in pattern 1-type individuals. In a similar case involving two cryptic marsh wren taxa, the preferentially amplified band was shown to be derived from the mitochondrial DNA, a high copy number element (D. Albright and M. Braun, unpublished obs.). Preferential amplification during RAPD reactions has little effect on the use of RAPD markers in diagnostic studies, but could be of serious importance in studies requiring independent markers, as in hybrid zone analysis, pedigree analysis, or relatedness estimates based on band sharing. In cases where independent markers are required, competition experiments could be performed to verify that variable bands produced by a single RAPD primer are amplified independently of one another. Finally, the inference that bands of similar size in different individuals are truly homologous should be made cautiously, and rejected in instances when complex banding patterns are produced, or the bands in question differ in intensity or reproducibility. Band homology can only be definitely determined by further investigation, such as Southern blotting or sequencing.

The ease with which we were able to obtain RAPD molecular markers more than compensated for any

characteristics of RAPD reactions which might complicate interpretation. With so many primers displaying potential as markers, we were able to focus on primers having particularly desirable characteristics as markers, such as strong diagnostic bands and simple patterns. Primers producing banding patterns that are in any way suspect should be passed over in favour of screening additional primers for markers with optimal characteristics. We found that a primary screen of primers on pooled DNA samples from the two species provided a convenient means for rejecting primers with bands that were polymorphic at intermediate frequencies in both species, without the need for running reactions on many individuals of each. Primers which pass this initial round of screening need to be tested on larger population samples, as some candidate marker bands were found to be present at low frequency in the opposing species when a greater number of individuals were analysed.

Our results using colony-maintained populations of well-characterized, yet morphologically cryptic, mosquito species portend that RAPD analysis will prove to be a powerful and technically accessible tool in elucidating the systematics of uncharacterized dipteran species complexes from natural populations. To perform RAPD analysis, researchers need possess only a very minimal wet laboratory, a PCR machine, a small agarose gel apparatus and power supply, and a UV light box and photography apparatus. The greater genetic variability of natural populations will complicate the search for completely diagnostic primers, but the minimal effort of screening additional primers suggests that this will not be an insurmountable problem. The ability of RAPD analysis to detect polymorphism holds great promise for detecting population structure and genetic differentiation even within well-established 'species' that would otherwise go unnoticed. Preliminary results of RAPD analysis of wild populations currently ascribed to *An. albiparvus* Lynch-Arribalzaga suggest that these populations actually derive from a minimum of three genetically distinguishable groups (R.C.W., unpublished data). Additionally, the ability to easily develop molecular markers for dipteran taxa holds promise for identification of species at all stages of development, permitting a rapid means of linking larvae, pupae and adults of uncharacterized species.

Experimental procedures

Source of specimens. Mosquitoes used in this study were obtained from colonies of *Anopheles gambiae* (G-3 strain) and *An. arabiensis* (GMAL strain) maintained by R. W. Gwadz at the Laboratory of Malaria Research, National Institute of Allergies and Infectious Diseases/National Institutes of Health, Bethesda, Maryland, USA. The identities of the species were confirmed using PCR primers supplied by J. Scott, W. Brogdon and F. Collins, Malaria Branch, Centers for Disease Control, Atlanta, Georgia, USA.

These diagnostic primer pairs were developed from rDNA sequence information, with one primer matching a highly conserved sequence common to all the species, and the other primer matching a species-specific sequence.

DNA isolation. Individual larvae or adults were ground with a plastic pestle in microcentrifuge tubes in 100 µl extraction buffer (100 mM Tris pH 8.0, 100 mM EDTA, 100 mM NaCl); proteinase K was then added to 200 µg/ml and SDS to 0.5%. After incubation at 55°C for 3–12 h, RNase was added to a final concentration of 100 µg/ml and incubated at room temperature for 30 min. The solution was extracted once with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, equilibrated with 10 mM Tris pH 8.0, 1 mM EDTA) by heating to 55°C for 10 min with periodic mixing of phases. After brief centrifugation in a microcentrifuge, the supernatant was extracted with chloroform/isoamyl alcohol (24:1) as above. The supernatant was collected, 2 volumes 95% ethanol were added to it, and the solution stored at –20°C for 15 min to precipitate the DNA. The DNA was pelleted (15,900 × g in a microcentrifuge for 4 min), washed with 70% ethanol, dried under vacuum, and dissolved in 100 µl 10 mM Tris pH 7.5, 1 mM EDTA. Typical yields were 0.5–6.5 µg DNA per individual.

RAPD PCR amplification. Detailed procedures are discussed in Bowditch *et al.* (1993). Total reaction volumes of 25 µl were used with the following final concentrations: 11 mM Tris-HCl (pH 8.3); 50 mM KCl; 1.9 mM MgCl₂; 0.1 mg/ml BSA; 0.1 mM each of dATP, dCTP, dGTP and TTP; 0.24 pmol/µl primer; 0.2–4.0 ng/µl template DNA; 0.02–0.06 U/µl Taq DNA polymerase.

PCR conditions. A Perkin-Elmer Cetus model 480 thermocycler was used for all reactions with the following parameters: 1 min denaturation at 94°C followed by 45 cycles of denaturation 1 min at 94°C, annealing 1 min at 35°C and elongation 2 min at 72°C, all with minimum ramp times.

Agarose gel electrophoresis. Using standard methods (Sambrook *et al.*, 1989), amplification products were analysed in 50 ml, 1.5% agarose minigels with 0.8 µg/ml ethidium bromide run at 50 V/25 mA for about 3 h in TBE (89 mM Tris base, 89 mM boric acid and 2 mM EDTA, pH 8.3). Amplification products were observed and photographed using long-wave (312 nm) ultraviolet light. Molecular weight standards were provided by lambda DNA digested with Hind III and ϕ X174 DNA digested with Hae III (New England Biolabs). The approximate molecular weight of amplification products was calculated using a program written for Lotus 1–2–3 by A. F. Cockburn, United States Department of Agriculture, Gainesville, Florida, USA.

Oligonucleotide primers. All primers screened were ten bases in length. Those with an 'P' prefix were synthesized in-house on a DNA synthesizer (Applied Biosystems model 391). Those primers with an 'A' or 'B' prefix were purchased from Operon Technologies, Alameda, Calif. Primers discussed in the text had the following sequences: P1, 5'-TGGTCAGTGA-3'; P2, 5'-TCTCGATGCA-3'; P4, 5'-CACATGCTTC-3'; P5, 5'-GCAAGTAGCT-3'; P6, 5'-TGGTCACTGT-3'; P14, 5'-AGGCGATAAG-3'; P37, 5'-TCACGATGCA-3'; A7, 5'-GAAACGGGTG-3'; B7, 5'-GGTGACGCAG-3'; B10, 5'-CTGCTGGGAC-3'. Primers P1, P2, P4, P5, P6 and P37 correspond to primers AP8g, AP4c, AP5h, AP6, AP8j and AP4 of Williams *et al.* (1990).

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